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# Antigenic sites of coxsackie A9 virus inducing neutralizing monoclonal antibodies protective in mice

Gabriele Buttinelli,[a] Valentina Donati,[a] Franco M. Ruggeri,[b] Paivi Joki-Korpela,[c] Timo Hyypia,[c] and Lucia Fiore[a,*]

[a] *Laboratory of Virology, Istituto Superiore di Sanità, Rome, 299-00161 Italy*
[b] *Laboratory of Ultrastructure, Istituto Superiore di Sanità, 299-00161 Rome, Italy*
[c] *Department of Virology, University of Helsinki, 00014 Helsinki, Finland*

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**Abstract**

A panel of murine IgG monoclonal antibodies (MAbs) was produced against coxsackievirus A9 (CAV9). Fifty-nine MAbs reactive in ELISA with purified CAV9 were identified. Eighteen of them could efficiently inhibit infection by CAV9 but not coxsackieviruses B. Neutralization-resistant CAV9 variants to four different MAbs were isolated and tested for resistance to neutralization by other MAbs of the panel. Three groups of reactivity including 10, 7, and 1 MAbs were thus identified. Sequencing of neutralization-escape virus mutants showed that neutralization by one MAb group was affected by change of VP3 amino acids 62 or 69. For the second group of reactivity, mutations included amino acids 154 or 165 of VP2. The only MAb of the third group selected for a change at residue 70 of VP2. Protection studies in a newborn mouse model of myositis suggested that either epitopes in VP2 or in VP3 mediate protection from CAV9 infection in vivo.


*Keywords:* Coxsackievirus A9; Monoclonal antibody; IgG; Immunity; Neutralization; Protection; Epitope

## Introduction

Enteroviruses are responsible for a wide range of human diseases, including poliomyelitis and other infections of the central nervous system, myocarditis, rashes, and respiratory infections, and there is evidence of their role in the development of insulin-dependent diabetes mellitus (IDDM) (Grist et al., 1978; Hovi et al., 1996; Hyoty et al., 1995). With the progression toward global poliovirus eradication, other clinically important members of the genus enterovirus are increasingly attracting the interest of scientists largely because research achievements on the widely studied polioviruses might help establish effective prophylactic measures. Among these pathogens is coxsackievirus A9 (CAV9), which can cause infections varying from common cold to fatal infections of the central nervous system (Grist et al., 1978; Kinnunen et al., 1987). Together with coxsackie B viruses, CAV9 is also implicated in the etiology of IDDM (Hyoty et al., 1995; Roivainen et al., 1998), a disease occurring frequently worldwide and leading patients to serious physical impairment and need for continuous therapy. Diabetes is not a normal outcome of CAV9 infection, and genetic and other factors are involved, such as certain HLA types and autoimmune disease and molecular mimicry (D'Alessio, 1992; Chatterjee, 1994; Baum et al., 1995).

Similar to the other enteroviruses which belong to the *Picornaviridae* family, CAV9 is a nonenveloped single-stranded RNA virus with an icosahedral capsid made of 60 copies each of four polypeptides, VP1 through 4 (Hendry et al., 1999). In CAV9, VP1 contains at its C-terminus a functional RGD-motif reacting with $\alpha_v\beta_3$-integrin during early virus–cell interactions (Roivainen et al., 1991, 1994), although the virus can also use an RGD-independent mode of entry (Hughes et al., 1995; Roivainen et al., 1996; Triantafilou et al., 1999). Despite the structural similarities

* Corresponding author. Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena 299-00161 Rome, Italy. Fax: +39-06-49902082.
*E-mail address:* fiore@iss.it (L. Fiore).

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with poliovirus, there is no conclusive evidence so far that the same regions in the capsid antigens of CAV9 would react with the host immune system eliciting neutralizing antibodies. A detailed map of neutralization determinants has been developed for all three types of poliovirus by characterization of wide panels of monoclonal antibodies (MAbs) and sequence analysis of neutralization-escape virus variants (Blondel et al., 1986; Diamond et al., 1985; Minor et al., 1985, 1986; Page et al., 1988; Wiegers et al., 1989, 1990). Complex epitopes, termed N-AgI through V, have thus been recognized involving amino acid regions of VP1, VP2, and VP3. Matching the results of these analyses with those of X-ray crystallographic studies has made it possible to identify the neutralization sites in the three-dimensional structure of the virion (Filman et al., 1989; Hogle et al., 1985). Participating in at least four of the sites known, VP1 appears to play a major role in the organization of poliovirus neutralization antigens, correlating well with its exposed position on the virion surface.

A recent antigenic analysis of CAV9 performed with polyclonal hyperimmune sera and a peptide scanning technique has revealed the presence of at least two immunogenic regions in VP1, one in VP2 and one in VP3 (Pulli et al., 1998a). Further studies of the C-terminal part of VP1 using peptide-specific antisera suggested a possible role of this region in virus neutralization (Pulli et al., 1998b). Interestingly, the most active amino acid region of CAV9 VP1 eliciting a neutralizing response appeared to include residues 293 through 302, which in poliovirus are adjacent to the VP1 moiety of N-AgIII (Page et al., 1988; Wiegers et al., 1990), suggesting that these viruses may share a similar molecular mechanism for neutralization. More recently, the determination of the atomic structure of CAV9 has shown that the RGD motif is located in a flexible extension on the south side of the "canyon," which for several members of the picornavirus family acts as the major site for attachment to cells (Hendry et al., 1999). To investigate these aspects further and to describe a more detailed map of CAV9 epitopes involved in antibody-mediated neutralization, we developed and characterized a library of monoclonal antibodies raised upon intraperitoneal inoculation of mice. Viral mutants resistant to neutralization by MAbs were selected, and their genome was sequenced to identify the mutations responsible for resistance and locate the antibody binding sites on the viral capsid proteins. Protection studies in newborn mice were performed to evaluate a possible activity of MAbs in protecting from CAV9 infection in vivo.

## Results

### *Development of immunity to CAV9 in mice and hybridoma screening*

Immune response to ip inoculation of mice with $10^5$ PFU of CAV9 (Griggs strain) was monitored by determining antiviral neutralizing antibodies in the sera collected after each antigen boost. Neutralizing antibody titers to CAV9 after the fourth inoculum ranged from 1:128 to 1:1024 among different animals (average Nt titer, 1:512). A mouse with high-antibody titer was used for fusion and hybridomas were screened by both neutralization in RD cells and ELISA assays. From $2 \times 10^8$ spleen cells yielding approximately 1500 vital clones, 53 original cell supernatants showed reactivity in ELISA and 16 were positive in a neutralization assay against CAV9. All neutralizing MAbs were also positive by ELISA and were confirmed to be IgG by an isotyping ELISA assay using class- and subclass-specific antisera to mouse immunoglobulins; no IgM antibody-producing clone was obtained. Two further neutralizing MAbs of six ELISA-positive clones were obtained from a second mouse fused separately. Hybridomas secreting neutralizing MAbs were recloned two to three times, and established antibodies were named K1 through K16 (first mouse), 6G11, and 4B3 (second mouse). All ELISA-positive MAbs were subsequently tested by a second neutralization assay on A549 cells, since these cells would allow us to recognize possible VP1 MAbs interfering with RGD-mediated virus binding, which might go undetected in the RD cell line. In fact, infection of A549 cells by CAV9 is dependent on the RGD motif, whereas RD cells can also be infected with mutants where this motif has been deleted (Hughes et al., 1995; Roivainen et al., 1996). However, none of the nonneutralizing MAbs in RD cells disclosed neutralizing activity toward CAV9 in this second assay.

### *Isolation of neutralization-escape CAV9 mutants*

To characterize in detail the viral epitopes recognized by the neutralizing IgG antibodies obtained, CAV9 variants resistant to neutralization by single antibodies were selected by isolation of viral plaques from RD cell monolayers infected with CAV9 in the presence of antibody. All isolated variants could be grown to similar titers as parental CAV9 during subsequent replication cycles in cell cultures, and all isolated variants retained their resistance characteristics upon passage in cell cultures.

### *Neutralization analysis of neutralization-escape mutants and other viruses with MAbs*

CAV9 natural mutants resistant to neutralization by MAbs were isolated to analyze the epitope specificity of the MAb panel obtained. Cross-neutralization tests performed with mutants vK3, vK6, vK8, and v4B3 corresponding to selecting MAbs K3, K6, K8, and 4B3 and the entire panel of MAbs developed allowed us to identify three different groups of reactivity among the 18 antibodies. In fact, MAbs K2, K3, K5, K7, K12, K16, and 6G11 (group 1) neutralized vK6, vK8, and v4B3 but not mutant vK3. Conversely, MAbs K1, K4, K6, K8, K9, K10, K11, K13, K14, and K15 (group 2) fully neutralized variants vK3 and v4B3, whereas

Table 1
Nucleotide and amino acid substitution in coxsackievirus A9 neutralization-resistant variants

| MAb group | CAV9 variant | Protein | Nucleotide change | Amino acid change |
|---|---|---|---|---|
| 1 | vK3.1 | VP3 | 1938 A-G | 69 Arg-Gly |
| | vK3.2 | VP3 | 1917 A-G | 62 Thr-Ala |
| | vK3.3 | VP3 | 1917 A-G | 62 Thr-Ala |
| | vK3.4 | VP3 | 1917 A-G | 62 Thr-Ala |
| | vK3.5 | VP3 | 1917 A-G | 62 Thr-Ala |
| 2 | vK6.1 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK6.2 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK6.3 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK6.4 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK6.5 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK6.6 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK6.7 | VP2 | 1410 G-A | 154 Glu-Lys |
| | vK6.8 | VP2 | 1410 G-A | 154 Glu-Lys |
| | vK8.1 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK8.2 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK8.3 | VP2 | 1410 G-A | 154 Glu-Lys |
| | vK8.4 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK8.5 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK8.6 | VP2 | 1443 A-G | 165 Lys-Glu |
| | vK8.7 | VP2 | 1443 A-G | 165 Lys-Glu |
| 3 | v4B3 | VP2 | 1159 A-T | 70 Lys-Met |

they had no effect on replication of mutants vK6 and vK8. Finally, MAb 4B3 neutralized the entire panel of variants except v4B3, so defining a distinct reactivity group (group 3). These results clearly indicated that the three antibody groups recognized three distinct epitopes spatially well separated on the virion. At the dilution used in this assay, all MAbs were able to efficiently neutralize the wild CAV9.

MAbs K3, K6, and 4B3, representative of the three groups, also neutralized the deletion mutant CAV9d12 in RD cells. Because CAV9d12 grew poorly on A549 cells, these assays were not performed. In cross-neutralization experiments with different human picornaviruses, MAbs K3, K6, and 4B3 neither neutralized coxsackie B viruses, serotypes 1 to 6, nor EV1 and HPEV1.

### *Sequence analysis of neutralization-escape mutants*

To investigate the protein specificity of the MAbs and to locate their binding site along the amino acid sequence and three-dimensional structure of CAV9, the virus variants isolated were subjected to nucleotide sequence analysis of the region encoding the capsid proteins (Table 1). The gene sequences determined for each mutant were compared with the sequence of the parental Griggs strain. To verify whether different mutations could be responsible for resistance to neutralization by the MAbs, viruses from several distinct plaques were picked from each selection plate, grown, and further purified separately before sequence analysis. Five variants selected against group 1 MAb K3, and eight variants derived from either MAbs K6 or K8 (group 2), were sequenced. Only one variant could be selected from group 3 MAb 4B3. As shown in Table 1, distinct neutralization-escape virus variants from both K6 and K8 MAbs showed either one of two base mutations, i.e., at nucleotide residues 1410 (G > A) or 1443 (A > G). These corresponded to amino acid changes in VP2, at locations 154 (Glu > Lys) and 165 (Lys > Glu), respectively. Differently, mutations identified in vK3 were at nucleotide residues 1917 (A > G) or 1938 (A > G), corresponding to amino acid changes in VP3, site 62 (Thre > Ala) or 69 (Arg > Gly), respectively. The virus variant to MAb 4B3 presented a mutation at position 1159 (A > T) corresponding to replacement of lysine with methionine at site 70 in VP2. None of the variants was shown to harbor mutations in VP1.

### *Analysis of hybridoma supernatants or immune sera with CAV9 or VP1 peptides by ELISA*

To investigate the apparent lack of immunogenicity of the VP1 N-terminus, we analyzed the hybridoma cell supernatants and the sera from mice immunized for MAbs production with immunogenic synthetic peptides corresponding to the flanking regions of the VP1 RGD motif (Pulli et al., 1998b), by ELISA. Sera from rabbits immunized against CAV9 or the VP1 peptides were also included. The results of these assays are reported in Table 2. None of the cell supernatants reacted with any peptide, whereas the serum from the mouse used for the first fusion, named M-CAV9, showed a weak antibody response against peptide 189, downstream of the RGD sequence.

### *Three-dimensional location of coxsackievirus A9 neutralization sites*

A three-dimensional representation of the CAV9 protomer is shown in Fig. 1. The yellow spheres in the image identify the positions of amino acids 62 and 69 of VP3, corresponding to the mutations of CAV9 variants escaping neutralization by MAb K3, whereas the orange spheres indicate VP2 amino acids 154 and 165, changed in mutants selected by both MAbs K6 and K8. The violet sphere shows

Table 2
Recognition of CAV9 or VP1 peptides by hybridoma supernatants or immune sera by ELISA

| Antibody | Peptides | | | |
|---|---|---|---|---|
| | p189 | p191 | CAV9 | PBS |
| MAbs (59 clones) | 0.14–0.26[a] | 0.15–0.22 | 2.10–2.60 | 0.14–0.24 |
| M-pre-immune | 0.09[b] | 0.20 | 0.20 | 0.10 |
| M-CAV9 | 0.70 | 0.30 | 2.50 | 0.10 |
| R-pre-immune | 0.20 | 0.20 | 0.22 | 0.15 |
| R-p189 | 2.60 | 0.50 | 1.70 | 0.30 |
| R-p191 | 0.38 | 2.50 | 0.45 | 0.18 |
| R-CAV9 | 0.70 | 0.40 | 2.30 | 0.18 |

[a] OD value range.
[b] OD value (450 nm). (R) rabbit serum; (M) mouse serum.

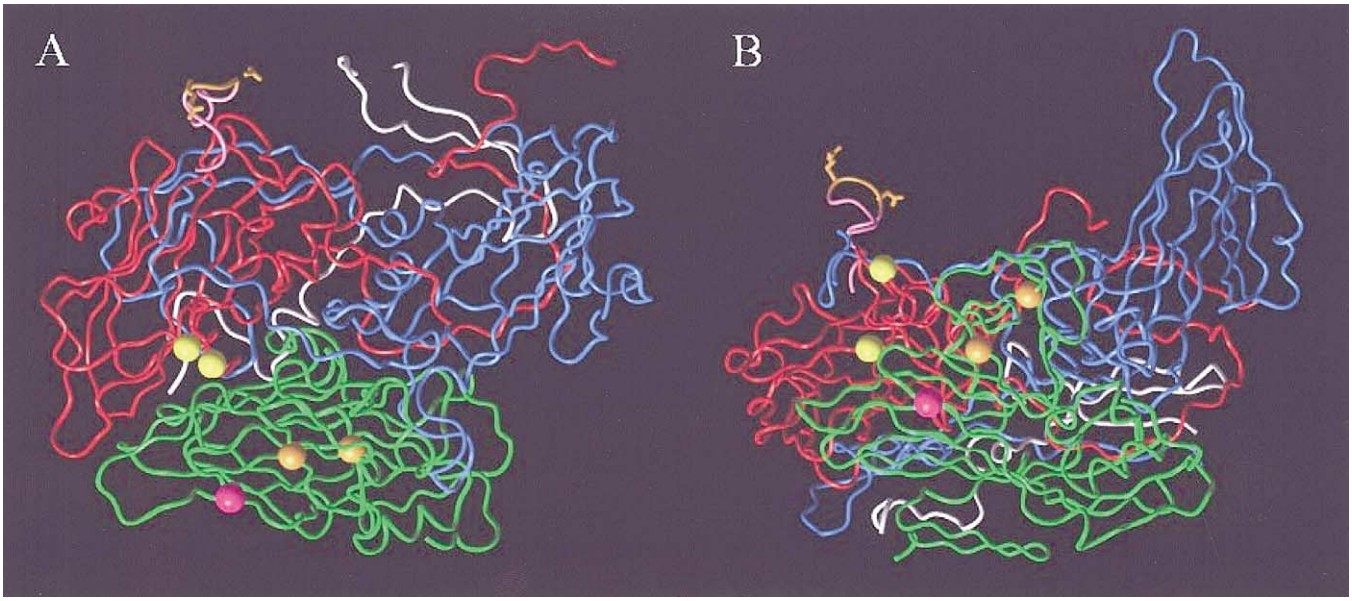


Fig. 1. Ribbon representation of the CAV9 (Griggs strain) protomer indicating the position of VP3 residues 62 and 69 (yellow spheres), and VP2 residues 154 and 165 (orange spheres) and 70 (violet sphere) identified in neutralization-resistant virus mutants. Views are from the front (A) and the side (B). VP1, VP2, VP3, and VP4 are colored in blue, green, red, and white, respectively. The C-terminal of VP2 RGD motif is shown in orange.

VP2 amino acid 70, targeted by MAb 4B3. The image, presented for description purposes, is based on the crystal structure defined for CAV9 by Hendry and co-workers (Hendry et al., 1999) and does not take into account possible perturbations induced by the change of the indicated amino acids. The C-terminal amino acid tail, containing the RGD motif and missing in the original sequence, was added to visualize the viral structure involved in integrin binding. The combination of the front (Fig. 1A) and side (Fig. 1B) views of the protomer shows that amino acid residues involved in both patterns of resistance to MAb neutralization are indeed closely related. The approximate distances estimated in the two cases were in fact 13 and 11 Å, respectively, for vK3 and vK6/vK8, in all cases within the spanning area of an IgG antibody. Conversely, the distance between the two pairs of residues as well as residue 70 of VP2 appeared to be higher, in the order of (25–35) Å, compatible with the absence of cross-reaction reported between MAb groups.

A comparison between the capsid proteins of CAV9 and poliovirus type 1 Mahoney is shown in Fig. 2. The epitopes identified in CAV9, VP3, and VP2 proteins appear to overlap, respectively, the antigenic sites N-AgIII (3b), and N-AgII (2b), and N-AgIV of poliovirus (Minor et al., 1986; Minor, 1990).

*Protection of infant mice from CAV9 infection by MAbs*

The ability of neutralizing MAbs to protect from infection in vivo was investigated in an infant mouse model of myositis (Harvala et al., 2002; Hyypia et al., 1993). Preliminary experiments showed that approximately 100 PFU of CAV9 corresponded to 1 $LD_{50}$ of CAV9 in infant mice inoculated ip at day 1 after birth. The age of mice at challenge appeared to be critical for a timely outcome of clinical signs and death (not shown); therefore, all protection experiments were standardized for virus challenge between 12 and 24 h after birth. Under these conditions, virtually 100% of mice died between 4 and 5 days after challenge. As shown in Table 3, treatment with representative MAbs from all groups proved to be highly effective in protecting mice from death. In the case of MAb K8, the calculated protection rate was slightly lower (66.7%) compared to the other MAbs. However, the lower rate essentially reflected the high mortality observed in just one of the litters (4/4) before day 2, which was likely due to problems of the dam rather than to an early virus effect.

## Discussion

Even though CAV9 and the other Coxackieviruses share similar biochemical characteristics and three-dimensional structure with the well-studied poliovirus, knowledge of their antigenic structure and pathogenic mechanisms is still incomplete. In particular, the absence of a detailed map of protective antigenic sites in coxsackieviruses is an obstacle to developing a safe immunologic prophylaxis against infection with these viruses. In fact, it has been suggested that CAV9 and CBV4 may cause type I diabetes (Hyoty et al., 1995; Roivainen et al., 1998), and CBV4 and CBV3 also may cause miocarditis (Grist et al., 1978; Hovi et al., 1996), possibly via the elicitation of an antibody or cell-mediated reaction against tissue antigens due to molecular mimicry of

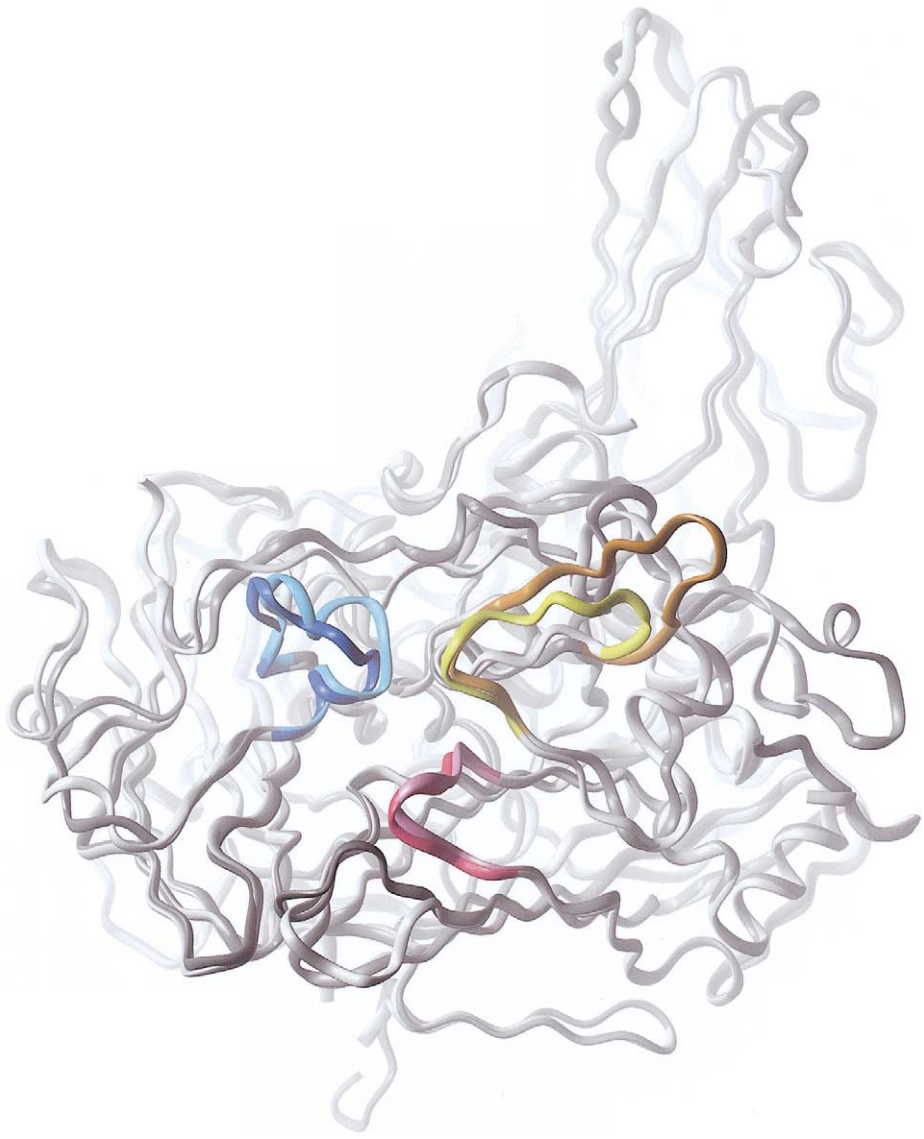

Fig. 2. 3D structure of poliovirus type 1 Mahoney and CAV9 protomers superimposed. Poliovirus Mahoney N-AgIII(3b) (cyan), N-AgII(2b) (yellow), and N-AgIV (violet) are compared with the epitopes of VP3 (blue) and VP2 (orange and red) identified in CAV9.

some viral proteins (Hyoty et al., 1995; Roivainen et al., 1998). A vaccine approach should therefore direct the immune response toward specific viral regions in the absence of antigenic sites involved on harmful reactions that might be fulfilled by developing recombinant peptide vaccines or DNA vaccines tailor-suited to present only the sequences needed to establish protection.

Table 3
Protection of newborn mice from CAV9 infection by MAbs

| MAb (group) | No. of mice challenged | No. of mice surviving at day: | | | | | | | | Percent of protection |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 15 | |
| K3 (1) | 15 | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 14 | 93.3 |
| K8 (2) | 15 | 14 | 10 | 10 | 10 | 10 | 10 | 10 | 10 | 66.7 |
| 4B3 (3) | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 100.0 |
| 3b[a] | 5 | 5 | 5 | 5 | 2 | 1 | 0 | 0 | 0 | 0.0 |
| None | 12 | 12 | 12 | 12 | 4 | 0 | 0 | 0 | 0 | 0.0 |

[a] 3b is a polio-specific MAb non-cross-reacting with CAV9.

In the case of CAV9, the knowledge of possible antigenic sites is to date limited to the studies of Pulli and co-workers (Pulli et al., 1998a,b), that in particular indicated the C-terminal end of the capsid protein VP1 as a putative important epitope eliciting neutralizing antibodies.

Our present study brings additional insight to the knowledge of CAV9 proteins associated with neutralizing antibody response, using the established approach of generating and characterizing monoclonal antibodies toward live virus in mice. Our data indicate that similarly to polioviruses CAV9 also induces a strong immune response in mice; in fact, a single fusion yielded as many as 53 distinct hybridoma clones secreting virus-specific MAbs.

The results of characterization of MAbs according to their pattern of reactivity with CAV9 neutralization-resistant mutants indicate that a majority of MAbs developed in this study are directed to VP2, and precisely, to an epitope which comprises amino acid residues 154 and 165. In fact, single mutations corresponding to either one of these positions caused the loss of neutralization by 10 of 18 MAbs studied. This suggests that VP2 of CAV9 may contain a reactive antigenic site which conversely was not observed in the study by Pulli and co-workers (Pulli et al., 1998a) using hyperimmune sera and peptide scanning. However, studies conducted with short peptides suffer from the limitation that peptides do not necessarily share the same spatial conformation as their amino acid sequence counterpart assembled in the virion, which may be critical for recognition by MAbs generated with assembled virions. In particular, for the more studied polioviruses it has been shown that in no case neutralizing monoclonal antibodies could react with denatured viral proteins in Western blotting, which implies that neutralization sites are conformation dependent (Minor et al., 1985, 1986; Page et al., 1988). In this view, our present approach presents the advantage that naive virions were used both to prime the immune system and to detect and characterize the ensuing immune response. A second neutralization epitope of CAV9, recognized by seven of our MAbs, appears to be located in VP3 between residues 62 and 69. This may be part of the same antigenic site located by Pulli and colleagues in the N-terminal region of VP3 (Pulli et al., 1998a). Finally, a third epitope was identified by a single MAb in VP2, residue 70.

Interestingly, the neutralization sites described by our present MAbs may be closely related to antigenic sites previously described for polioviruses. In fact, our MAbs K6 and K8 selected mutations in VP2 residues 154 and 165 of CAV9. The latter in poliovirus is within neutralization site N-AgII, comprising VP2 residues 164–170 (Minor et al., 1986; Page et al., 1988; Wiegers et al., 1990). Conversely, the VP3 moiety of poliovirus site N-AgIII includes among others residues 58–61, 66, 70–73 (Minor et al., 1986; Page et al., 1988), while our CAV9 MAb K3 is targeted to VP3 residues 62 and 69. This coincidence at the level of amino acid sequence also finds correspondence between the protein tertiary structure of CAV9 and polio capsid proteins, despite the deletion present in the "puff" region of CAV9 VP2 as compared with polio site 2b (data not shown). The similarities found between CAV9 and poliovirus are particularly suggestive, since in poliovirus type 1 these residues of N-AgIII and N-AgII may be also relevant in the elicitation of mucosal immunity (Fiore et al., 1997). Also the third epitope identified in VP2 (aa 70) with MAb 4B3 recalls a known site of poliovirus, namely N-AgIV (aa 72 of VP2, 76, 77, and 79 of VP3).

The computer-assisted 3D localization in the capsid of the VP2 and VP3 epitopes identified in the present study corroborates the absence of reactivity between the three groups of MAbs described. In fact, the calculated distance between residues 154–165 and 70 of CAV9 VP2 and residues 62–69 of VP3 approximates (25–35) Å, that is, enough to consider unlikely that these residues belong to the same antibody binding site. Conversely, the short distance between either VP2 residues 154 and 165 or VP3 residues 62 and 69 is consistent with the cross-reactivity exhibited within these two groups of MAbs.

Combined results of sequence comparison and 3D analysis confirm that both VP3 and VP2 epitopes appear to be exposed on the CAV9 virion surface. In particular, residues 3062–3069 are apparently located in the prominent "knob" structure (residues 58–69 of VP3) characterized by a high variability among picornaviruses (Hendry et al., 1999), whereas VP2 amino acids 154–165 reside in the puff region. This latter encompasses the E–F loops (residues 129–178), and in poliovirus is implicated in receptor binding (Hendry et al., 1999). Both regions are therefore well fitted for the accomplishment of an antiviral effect by bound antibodies. Also, residue 70 of VP2 is located in the B–C loop of VP2.

In addition to neutralization in cell cultures, the three antigenic sites described by our MAb panel may be also involved in protection from CAV9 infection in vivo. In fact, three representative MAbs targeting the three VP2 and VP3 epitopes were shown to efficiently reduce mortality in infant mice challenged with a lethal dose of CAV9. These results with single epitope-specific antibodies are encouraging in terms of developing a vaccinal prophylactic approach to CAV9 infection. Also, they demonstrate the suitability of the newborn mouse model for studying in detail the molecular basis of the protective immune response to coxsackievirus.

Of the 18 neutralizing MAbs characterized in this study, none was directed to VP1. This finding is apparently in contrast with previous reports that CAV9 VP1 displays at least two epitopes, one of which is probably involved in neutralization (Pulli et al., 1998a,b). More precisely, these authors reported that the C-terminus of VP1 contains an RGD motif that has been related to the ability of the virus to infect some permissive cells via $\alpha_v\beta_3$-integrin binding (Roivainen et al., 1994). In such a case, blocking of this viral site by an antibody would presumably prevent virus binding to the cell surface and consequently infection. For this reason, our hybridomas were screened for neutralization both on the RD cell line displaying a second receptor other than $\alpha_v\beta_3$-integrin for virus binding and on A549 cells which present only $\alpha_v\beta_3$-integrin as receptor for CAV9 (Roivainen et al., 1996). Nonetheless, the use of the latter cells did not show further neutralizing MAbs in addition to the ones detected on RD cells, ruling out the possibility that MAbs to VP1 might have gone undetected due to the presence of a subsidiary receptor (Roivainen et al., 1996). While we failed to isolate neutralizing MAbs to VP1, we did however detect a weak reactivity versus the RGD flanking region of CAV9 VP1 for the immune mouse serum in a peptide-based ELISA assay. Identical results were obtained

by immunizing a rabbit with CAV9. The amino acid sequence toward which CAV9 immunization apparently elicited an immune response spans VP1 residues 293 through 302, beginning from the amino acid immediately following the RGD motif. The lack of reactivity of the same sera with a second peptide corresponding to VP1 stretch 272–287 also suggests that the RGD stretch itself is not part of the antibody binding site. Our results with peptides confirm those reported previously by Pulli and co-workers (Pulli et al., 1998a,b). Although the RGD motif involved in coxsackievirus binding to cells is contiguous to the 293–302 VP1 region, these authors suggested that antibodies targeted to this peptide would unlikely act by steric hindrance on the $\alpha_v\beta_3$-integrin-binding domain of CAV9. In fact, their anti-peptide antiserum could also neutralize virus growth in RD cells, which are infected by CAV9 in an RGD-independent manner (Roivainen et al., 1996). Rather, the authors suggested that interference with uncoating or aggregation of virions may be the mechanisms by which these specific antibodies play their role, as previously reported for other picornaviruses (Hewat and Blaas, 1996; Smith et al., 1993; Thomas et al., 1985). In FMDV, the RGD sequence has been shown to be part of a neutralization epitope (Fox et al., 1989) and, more specifically, to block the antiviral activity of antibodies preventing FMDV binding to BHK hamster kidney cells.

Although our findings seem to indicate that CAV9 VP1 may be poorly immunogenic in mice upon parenteral immunization, we cannot exclude that this protein may represent an important viral antigen in neutralization and protection in vivo. In fact, we presently generated MAbs by parenteral immunization of adult mice which are not expected to permit an extensive replication of CAV9 as in the course of natural infection. Although the procedure we used has been widely reported for other viruses (Blondel et al., 1986; Diamond et al., 1985; Greenberg et al., 1983; Minor et al., 1986; Page et al., 1988), it is possible that a different route of uptake and processing of the virus may lead to a different epitope targeting by the immune system. Further work is therefore needed to address this issue. Also, our study confirms that mechanisms other than inhibition of binding to $\alpha_v\beta_3$-integrin are involved on CAV9 neutralization.

In our experiments, no significant cross-reactivity was observed between different representatives of human picornaviruses when the neutralization test was performed with CAV9 MAbs. Previously, CAV9 rabbit hyperimmune serum was shown to inhibit infectivity of HPEV1, a picornavirus sharing a similar RGD motif in VP1 with CAV9 (Joki-Korpela et al., 2000). This phenomenon was not observed with a CAV9 RGD deletion mutant or with CBV4, which has no RGD motif, suggesting that the antigenic determinant responsible for the cross-neutralization would be located in the C-terminus of VP1. In the present work, MAbs K3, K6, and 4B3 specifically inhibited the growth of CAV9 and the RGD deletion mutant CAV9-12d, indicating that the neutralizing effect of these antibodies was directed toward structures which are separate from antigenic regions in the VP1 RGD-containing region. Also, the fact that the growth of HPEV1 was not inhibited by these antibodies supports this idea.

In conclusion, our study indicates that CAV9 is a potent stimulator of the antibody-oriented immune response in mouse that does not react differently than poliovirus. It also highlights the targeting of VP2 and VP3 CAV9 capsid proteins by the immune system, more specifically, epitopes that are spatially related with neutralization sites N-AgII (2b) and N-AgIII (3b) described for poliovirus. As suggested for poliovirus N-AgII and N-AgIII residues, CAV9 neutralization sites identified herein might also be relevant in the elicitation of mucosal immunity (Fiore et al., 1997), therefore, representing a possible ideal target for vaccination.

## Materials and methods

### *Cells, viruses, and virus-derived peptides*

Human rhabdomyosarcoma cells (RD, ATCC) were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) at 37°C. Human lung carcinoma cells (A549, ATCC) were grown in Ham F12 medium containing 10% FBS. The virus used for production of monoclonal antibodies was the Griggs strain of CAV9 (Chang et al., 1989) and was amplified after initial infection of cells with infectious cDNA. Coxsackieviruses B1–B6, Echovirus 1 (EV1) Farouk strain, human parechovirus 1 (HPEV1) Harris strain (from ATCC), and CAV9-derived virus mutant presenting deletion in the RGD motif and its flanking regions (CAV9d12; aa 282–288, TTVANTH) (Hughes et al., 1995) were used in neutralization assays (see below) to test possible cross-reactions of anti-CAV9 MAbs. Synthetic peptides covering the flanking regions of the RGD motif of CAV9 VP1, namely PAM 189 (aa 293–302, MSTLNTHGAF) and PAM 191 (aa 272–287, TDTRKDINTVTTVAQS) (Pulli et al., 1998b), and rabbit sera elicited against these peptides were used in ELISA assays (see below).

### *Virus purification*

Lysates from cell cultures infected with CAV9 were ultracentrifuged at 35,000 rpm in a Beckman Ti35 rotor for 3 h at 4°C. Pellets were resuspended in phosphate-buffered saline (PBS) and layered onto a 30% sucrose cushion in PBS. After centrifugation in a Beckman SW 41 rotor at 35,000 rpm for 2 h at 4°C, the virus pellet was further purified by isopycnic banding in a CsCl gradient using a Beckman SW 41 rotor at 35,000 rpm for 20 h at 16°C. The viral band was then resuspended in 1 to 2 ml of PBS, dialyzed against PBS, and used for either immunization of

mice or coating of ELISA plates. Virus infectious titers were determined in cell cultures prior to immunization.

*Immunization of mice*

Six week-old female BALB/c mice (supplied by Charles River) were inoculated ip with 500 μl of purified CAV9 (containing $10^5$ PFU) in the presence of Freund's complete adjuvant. Mice were boosted three times at 3-week intervals with incomplete adjuvant; approximately 10 days after boosting, sera from immunized animals were tested for the presence of neutralizing (Nt) antibody to CAV9. The average Nt antibody titer was approximately 1:512 after the fourth injection. Sera were also tested for the presence of antibodies against synthetic peptides or viruses by ELISA.

*Fusion*

Mice received a final boost 4 days before fusion, by intravenous injection of $10^5$ PFU of virus. One of the animals was sacrificed, and the spleen was resected and prepared for fusion as described previously (Greenberg et al., 1983). Hybridoma cell supernatants were screened using a microneutralization test and an ELISA assay (see below). Selected cultures were cloned at least twice by limiting dilution using a mouse thymocyte feeder layer, and where requested, MAbs were amplified in mouse ascites. Determination of the MAb immunoglobulin class was performed by ELISA using a mouse antibody isotyping kit (Pierce, Rockford, IL). IgG MAbs were purified from ascites fluid by affinity chromatography using protein G (ImmunoPure G IgG Purification Kit, Pierce) according to the manufacturer's instructions. Concentration of purified immunoglobulins was determined by the Bio-Rad Protein Assay.

*Enzyme-linked immunosorbent assay (ELISA)*

Microtiter ELISA plates were coated overnight at 4°C with 50 μl/well of purified CAV9 diluted 1:200 in PBS and blocked with 5% FBS in PBS for 1 h at 37°C. Wells were incubated with a 1:10 dilution of hybridoma cell supernatants in 1% BSA/PBS for 2 h at 37°C. Plates were subjected to secondary incubation with an anti-mouse IgG (H+L) antibody conjugated with alkaline phosphatase (AP). Reaction was developed with *p*-nitrophenol phosphate (Sigma 104) in 10 mM diethanolamine, pH 11, and optical densities were measured at a wavelength of 405 nm, after 1 h incubation at 37°C. For peptide ELISA assays, peptides PAM 189 and PAM 189 and PAM 191 (10 μg/ml) were diluted 1:6000 in carbonate buffer (0.04 M, pH 9.6) and incubated onto ELISA plates overnight at 4°C, and plates were blocked with buffer containing 5% FBS for 2 h at 37°C. After washing with PBS–Tween (0.025%; PBS/T), wells were incubated with rabbit anti-peptide-specific antisera or negative preimmune sera, diluted 1:1000 in PBS/T or with hybridoma supernatants, diluted 1:5, for 1 h at 37°C. Secondary incubation was performed with anti-rabbit or anti-mouse IgG (H+L) HRP-conjugated antibodies diluted 1:100 in PBS/T/FBS for 1 h at 37°C. Reactions were developed with TMB Kit (Vector), and optical density was measured as described above.

*Neutralization assays*

A standard microneutralization assay was used (Weber et al., 1994). Approximately 100 $TCID_{50}$ of virus were mixed with either a 1:10 dilution of hybridoma cell supernatants for screening of the fusion or serial dilutions of selected antibodies, and the mixtures were incubated for 60 min at 37°C prior to inoculation onto RD or A549 cells in 96-well plates. With a similar procedure, antigenic virus variants (see below) were tested for cross-neutralization by MAbs. Monolayers were scored for CPE daily for 4 days. Neutralizing titers were expressed as the dilution of cell supernatant or ascites fluid resulting in the absence of CPE in 50% of replica cultures.

*Cross-neutralization of CAV9 hybridoma supernatants against different human picornaviruses*

To study the cross-neutralizing activity of the obtained MAbs among human picornaviruses, we tested the ability of representative neutralizing MAbs to inhibit the infectivity of CAV9, CAV9d12 (VP1 C-terminal deletion mutant), echovirus 1 (EV1), and human parechovirus 1 (HPEV1) in RD and A549 cells. Fifty microliters of undiluted MAb was combined with an equal amount of virus dilution (100 PFU of each virus) and incubated for 1 h at 37°C, and 100 μl of mixture was added onto washed cells. Each antibody was tested in four parallel wells. After 15 min incubation at RT, the medium was replaced by 0.5% carboxy methyl cellulose in culture medium. Following incubation at 37°C for 48 h, the cells were stained with crystal violet prior to counting the number of plaques.

*Isolation of CAV9 antigenic variants*

Neutralization-resistant CAV9 variants were selected in RD cell monolayers by plaquing serial dilutions of virus ($10^7$ to $10^8$ PFU) previously neutralized with the selected MAbs (1:100 dilution of ascites fluid), and including the antibody (1:1000) in the overlay medium during growth. Several single well-developed viral plaques were picked from each plate and subjected to at least two further cycles of plaque purification before cell-culture expansion in antibody-free medium. Identification of neutralization-escape CAV9 mutants was eventually confirmed by neutralization tests. Virus mutants were designated with the name of the corresponding MAb used for selection preceded by the type "v."

*Nucleotide sequencing*

The capsid region of CAV9 variants was sequenced by using RT-PCR. A few microliters of viral suspension were resuspended in RT buffer containing 20 mM Tris–HCl pH 8.8, 75 mM KCl, and 2.5 mM $MgCl_2$. After heat denaturation at 70°C, genomic RNA was subjected to reverse transcription and cDNA amplification with a panel of synthetic oligonucleotides as primers. Amplified products were purified and sequenced by the DyeDeoxy Terminator Cycle Sequencing Kit (Applied BioSystems, Perkin–Elmer, Foster City, CA) with the same primers used for amplification. RNA from CAV9 Griggs parental strain was included as control. The sequences obtained were aligned with the reference strain sequences.

*Structural analysis of coxsackievirus A9 antigenic sites*

Structural analysis was performed by using the Insight II molecular modeling package (MSL, San Diego, CA) (Bernstein et al., 1977) using the coordinates of the CAV9 reported by Hendry and co-workers (PDB code: 1DM4) (Hendry et al., 1999). To visualize the RGD motif localization, the structure was integrated with the C-terminal sequence by a homology modeling approach, based on the crystal structure of the RGD motif of the foot and mouth disease virus (FMDV) (PDB CODE: 1QJG) (Hewat et al., 1997). Comparison of poliovirus and CAV9 antigenic sites was performed by superimposing the promoter 3D structures. Poliovirus type 1 Mahoney coordinates reported by Hogle and co-workers were used (Hogle et al., 1985).

*Protection studies in mice*

An in vivo model of myositis in the suckling mouse was used (Harvala et al., 2002; Hyypia et al., 1993). To assess the activity of MAbs in vivo, a procedure similar to that described by Pincus et al. (1995) for murine leukemia virus was used. BALB/c mice within 24 h from birth were injected ip with 50 $\mu$g of selected purified MAbs in 50 $\mu$l PBS. After 3 h, mice were challenged with 50 $\mu$l of virus suspension containing approximately 100 $LD_{50}$ of CAV9. To titrate virus infectivity in the mouse, preliminary experiments were performed with serial dilutions of virus and showed that 1 $LD_{50}$ equaled approximately $10^2$ PFU. To induce a prolonged presence of MAb in the body fluids, MAbs were also administered at day 2 and 4. Test mice were followed for up to 15 days after challenge, and protection was defined as the absence of a fatal outcome. Values reported represent pooled results from two independent experiments.

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